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Requirement of the transmembrane semaphorin Sema4C for myogenic differentiation

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Abstract Semaphorins constitute a large family of signaling proteins that contribute to axonal guidance. Here we demonstrate that the transmembrane semaphorin Sema4C is up-regulated both in the early stage of differentiation of C2C12 mouse skeletal myoblasts into myotubes and during injury-induced muscle regeneration in vivo. Depletion of Sema4C in C2C12 cells resulted in marked attenuation of myotube formation. A fusion protein containing the extracellular Sema domain and a peptide corresponding to the intracellular COOH-terminal region of Sema4C each inhibited the differentiation of C2C12 cells. These findings indicate that Sema4C-mediated interaction among myoblasts plays an important role in terminal myogenic differentiation.

Keywords: C2C12 cell; Muscle regeneration; Myogenic differentiation; Myogenin; Semaphorin; Sema4C

1. Introduction

Myogenic differentiation is a highly orchestrated and multistep process [1]. During terminal myogenic differentiation, myoblasts exit the cell cycle, increase their expression of myogenic transcription factors such as MyoD and myogenin, and then form multinucleated myotubes and initiate the expression of myofibrillar proteins such as myosin heavy chain (MHC) [2,3]. In addition to myogenic transcription factors, several proteins including integrins [4] and cadherin [5] have been implicated in regulation of terminal myogenic differentiation.

Members of the semaphorin family regulate axonal guidance in the developing nervous system [6]. They include both secreted and membrane-bound molecules, the common structural feature being the presence of an extracellular Sema domain that comprises a stretch of ~500 amino acids. Among vertebrate transmembrane semaphorins, Sema4B [7] and Sema4C [8] possess a PSD-Dlg-ZO-1 (PDZ) domain-binding motif in their cytoplasmic COOH-terminal regions. Semaphorins elicit intracellular responses through their receptors, which include neuropilins and plexins [9,10], whereas Sema4B and Sema4C are also thought to mediate intracellular signaling through interaction with a PDZ domain-containing protein.

In addition to neuronal development, semaphorins have been implicated in processes such as organogenesis and immune responses [11,12]. We have now examined the role of Sema4C in myogenic differentiation of myoblasts into myotubes with the use of the mouse skeletal muscle C2C12 cell line. We show that Sema4C-mediated intercellular interaction among myoblasts is required for terminal myogenic differentiation.

2. Materials and methods

2.1. Cell culture

C2C12 cells were maintained in growth medium, comprising DMEM supplemented with 20% fetal bovine serum. Differentiation of the myoblasts into myotubes was induced by switching the culture medium to the differentiation medium (DM), consisting of DMEM supplemented with 2% horse serum.

2.2. RNAi

Control and Sema4C mRNA-specific siRNAs were obtained from Qiagen (Valencia, CA) in duplex form. The target sequences were as follows: Cont-siRNA, 5′-AATTCTCCGAACGTGTCACGT-3′ Sema-siRNA-1, 5′-GCGGATGAATTACGACGGAAA-3′; and Sema-siRNA-2, 5′-CAGGACCTGCCTGCAGAACAA-3′. C2C12 cells in 24-well plates were transfected with siRNAs (1 μg per well) with the use of RNAiFect (Qiagen); the first transfection was performed when the cells had achieved 50–60% confluence, and second and third transfections were performed at intervals of 24 h (when the cells were 70–80% and ~100% confluent, respectively). The second and third transfections were performed in DM.

2.3. Immunofluorescence analysis

C2C12 cells were grown on 15-mm cover glasses coated with 0.2% gelatin and were fixed for 15 min with 4% paraformaldehyde. The cells were permeabilized with 0.5% Triton X-100, incubated for 30 min with 1% bovine serum albumin, and then exposed consecutively to a mouse monoclonal antibody to skeletal muscle MHC (Sigma, St. Louis, MO) and Alexa Fluor 488-conjugated goat antibodies to mouse immunoglobulin G (Molecular Probes, Eugene, OR). In some experiments, the cells were double stained with DAPI.

2.4. RT-PCR

The PCR protocol was designed to maintain amplification in the exponential phase. The sequences of the PCR primers were as follows: Sema4C sense, 5′-CACCCGGCGACTTCGAGAAGAGCTAGAAA-3′; Sema4C antisense, 5′-TCATACTGAAGACTCCTCTGGGTTG-3′; MyoD sense, 5′-GATGGCATGATGGATTACAGC-3′; MyoD antisense, 5′-GACTATGTCCTTTCTTTGGGG-3′; myogenin sense, 5′-GCTCAGCTCCCTCAACCAG-3′; myogenin antisense, 5′-ATGTGAATGGGGAGTGGGGA-3′; G3PDH (internal control) sense, 5′-ACCACAGTCCATGCCATCAC-3′; G3PDH antisense, 5′-TCC-

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Abbreviations: MHC, myosin heavy chain; PDZ, PSD-Dlg-ZO-1; DM, differentiation medium; GST, glutathione *S*-transferase

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ACCACCCTGTTGCTGTA-3′; Sema4B sense, 5′-AGACTCTGTGGTATGAGAGCTGACT-3′; Sema4B antisense, 5′-GTGGCTATGTTGTAGTCTTCCTTGT-3′.

2.5. Preparation of Sema4C fusion proteins and synthetic peptides

Complementary DNAs encoding the Sema (amino acids 46–489), Psi-repeat (490–562), and Ig (564–665) domains of mouse Sema4C were cloned into pDEST15 (Invitrogen, Carlsbad, CA). The resulting plasmids were introduced into *Escherichia coli* BL21-AI, and expression of the GST fusion proteins was induced by 0.2% L-arabinose. The recombinant proteins were then purified with the use of glutathione–Sepharose beads (Amersham, Piscataway, NJ).

Peptides were synthesized by Genemed Synthesis (South San Francisco, CA) and purified by high-performance liquid chromatography. The peptides contained a modified TAT sequence (YARAAARQARA) [13] followed by the COOH-terminal sequence of Sema4C (QPLPDSNPEESSV) (TAT-W) or by a corresponding mutated sequence (QPLPDSNPEEASA) (TAT-M).

2.6. Muscle regeneration model

Mice (BALB/c, 12 weeks old) were anesthetized by intraperitoneal injection of pentobarbital. Injury and regeneration of skeletal muscle were induced by intramuscular injection of 400 μl of 10 μM cardiotoxin (Latoxan, Valence, France) into the hind limbs, as described previously [14]. Treated muscles were examined before and at various times after injury, with three animals per time point. These experiments were approved by the Animal Ethics Committee of Yamaguchi University School of Medicine.

3. Results

3.1. Up-regulation of Sema4C during myogenic differentiation of C2C12 cells

The differentiation of C2C12 myoblasts into myotubes was induced by removal of growth factors from the culture medium. Abundant myotubes were observed 5 days after switching to DM (Fig. 1A). Immunoblot and RT-PCR analyses revealed the presence of small amounts of Sema4C protein (Fig. 1B) and Sema4C mRNA (Fig. 1C) in C2C12 cells before the induction of differentiation. The expression of Sema4C was up-regulated at both the protein and mRNA levels after transfer of the cells to DM. The up-regulation of Sema4C expression occurred before that of myogenin expression and concomitantly with that of MyoD expression (Fig. 1B and C). The expression of Sema4C subsequently decreased as mature myotubes formed. We analyzed the expression of Sema4B by RT-PCR only, given that specific antibodies are not available. Sema4B mRNA was detected in myoblasts, but its abundance was not affected by the induction of differentiation (Fig. 1C).

3.2. Depletion of Sema4C by RNAi inhibits myotube formation

To clarify the role of Sema4C in terminal myogenic differentiation, we examined the effects of its depletion by RNA interference (RNAi). Transfection of C2C12 myoblasts with either of two different small interfering RNAs (Sema-siRNA-1, Sema-siRNA-2) specific for Sema4C mRNA resulted in significant inhibition of myotube formation and MHC expression (Fig. 2A and B). A control siRNA had no such effects. Immunoblot analysis revealed that both Sema4C siRNAs, but not the control siRNA, largely blocked the up-regulation of Sema4C (Fig. 2C and D). The two Sema4C siRNAs also inhibited the increase in the expression of myogenin, but not that in the abundance of MyoD (Fig. 2C and D). The transcriptional activity of MyoD was not significantly changed by the Sema4C siRNAs (data not shown) when examined by a luciferase assay using the MyoD-dependent reporter 4Rtk-luc (kindly provided by Dr. A. Lassar, Harvard Medical School), which contains four tandem E boxes from the muscle creatine kinase enhancer upstream of the thymidine kinase basal promoter [15]. These two Sema4C siRNAs have no significant effect on the expression of Sema4B when analyzed by RT-PCR (data not shown). On the other hand, overexpression of Sema4C in myoblasts with the use of an adenoviral vector had no marked effect on the differentiation of myoblasts into myotubes (data not shown).

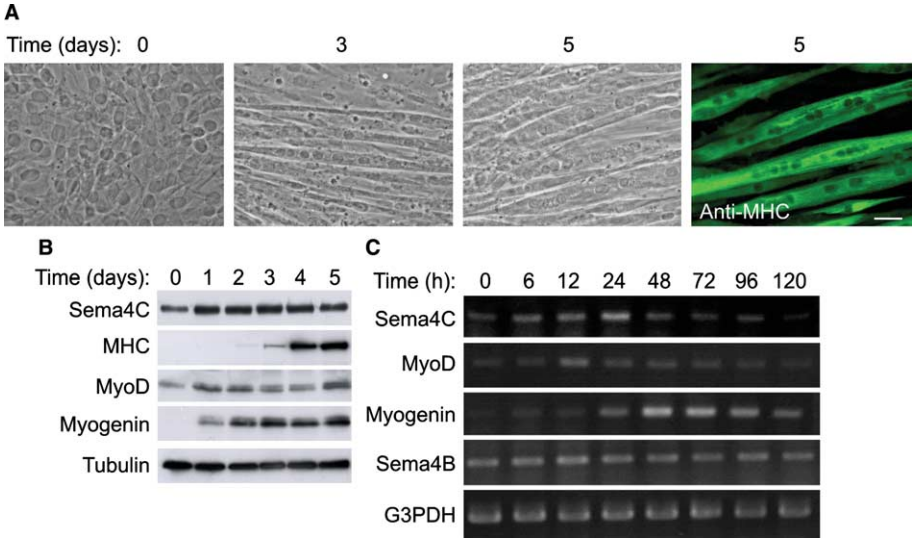

Fig. 1. Expression of Sema4C during differentiation of C2C12 myoblasts into myotubes. (A) C2C12 myoblasts (>70% confluent) were induced to differentiate by culture in DM. Phase-contrast microscopic images of the cells at 0, 3, and 5 days as well as an immunofluorescence image obtained with antibodies to MHC at 5 days after induction of differentiation are shown. Bar, 25 μm. (B) Cell lysates prepared at the indicated times after induction of differentiation were subjected to immunoblot analysis with antibodies to the indicated proteins. (C) Total RNA prepared from cells at the indicated times after induction of differentiation was subjected to RT-PCR analysis of the indicated mRNAs.

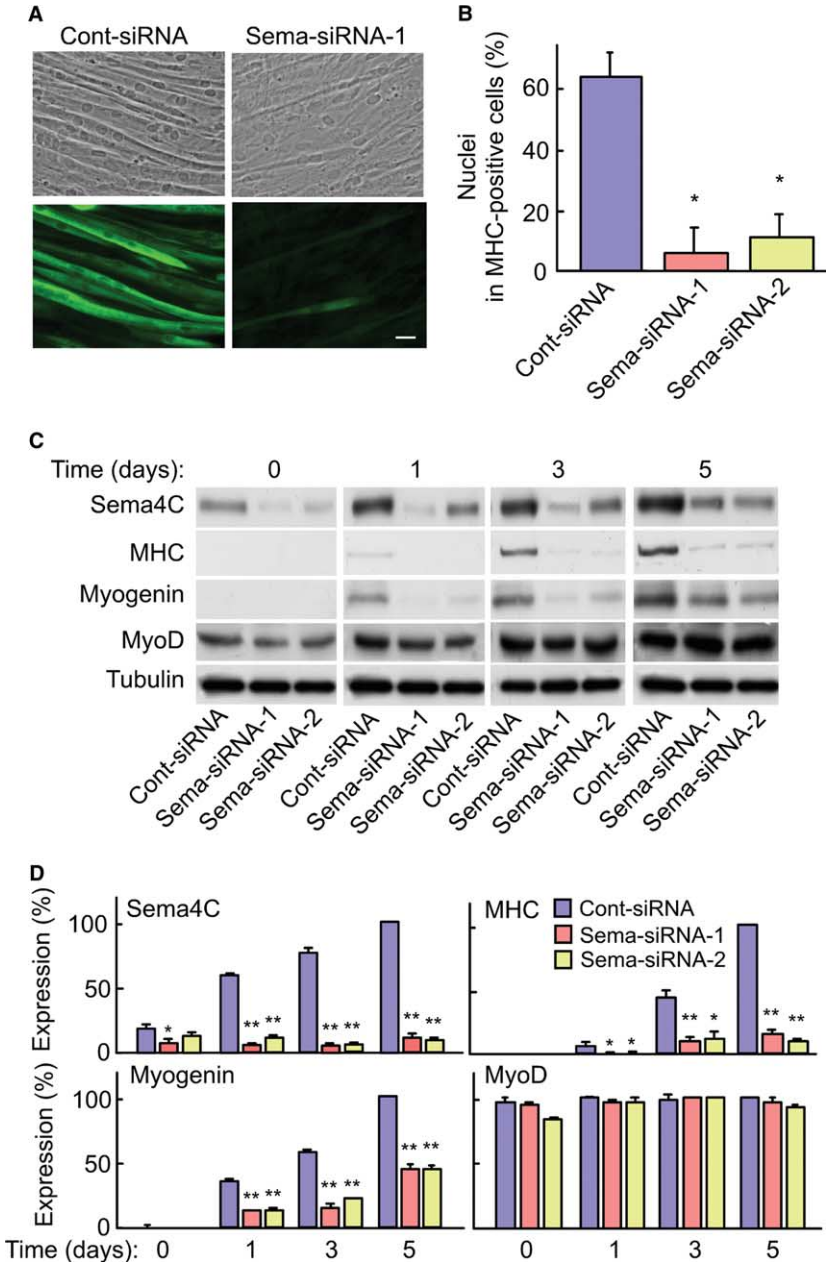

Fig. 2. Effects of Sema4C depletion on the differentiation of myoblasts. (A) C2C12 cells were transfected with a control siRNA (Cont-siRNA) or with either of two siRNAs specific for Sema4C mRNA (Sema-siRNA-1, Sema-siRNA-2) and were induced to differentiate by culture in DM. The cells were examined by phase-contrast microscopy (upper panels) or subjected to immunofluorescence analysis with antibodies to MHC (lower panels) 5 days after induction of differentiation. Bar, 25 μm. (B) Nuclei were visualized with DAPI. The percentage of nuclei in MHC-positive cells in each of 10 randomly chosen fields (magnification 200×) was determined at this time to quantify myotube formation. Data are means ± S.E. from a representative experiment; $^{*}P < 0.001$ versus the value for cells transfected with the control siRNA. (C) Cell lysates were subjected to immunoblot analysis at the indicated times after induction of differentiation. (D) The expression of each protein was quantified by scanning blots similar to those shown in (C) was normalized by the expression level of tubulin, and was expressed as a percentage of the maximum normalized value. Data are means ± S.E. from three independent experiments; $^{*}P < 0.01$, $^{**}P < 0.001$ compared with the value for cells transfected with the control siRNA.

3.3. The extracellular Sema domain of Sema4C is important for myogenic differentiation

The extracellular region of Sema4C contains Sema, Psi-repeat, and immunoglobulin domains. We then examined the possible effects of GST fusion proteins containing each of these domains individually (GST-Sema, GST-Psi, GST-Ig) on C2C12 differentiation. The addition of GST-Sema, but not that of GST, GST-Psi, or GST-Ig, to DM inhibited both the formation of myotubes (Fig. 3A and B) and the up-regulation of myogenin (Fig. 3C and D), without affecting the expression of Sema4C (Fig. 3C and D). These effects of GST-Sema were thus likely attributable to a dominant negative action.

3.4. Role of the intracellular COOH-terminus of Sema4C in myogenic differentiation

Given that Sema4C has a PDZ domain-binding motif (SSV) at its cytoplasmic COOH-terminus, we next examined whether the interaction of this motif with a PDZ domain-containing

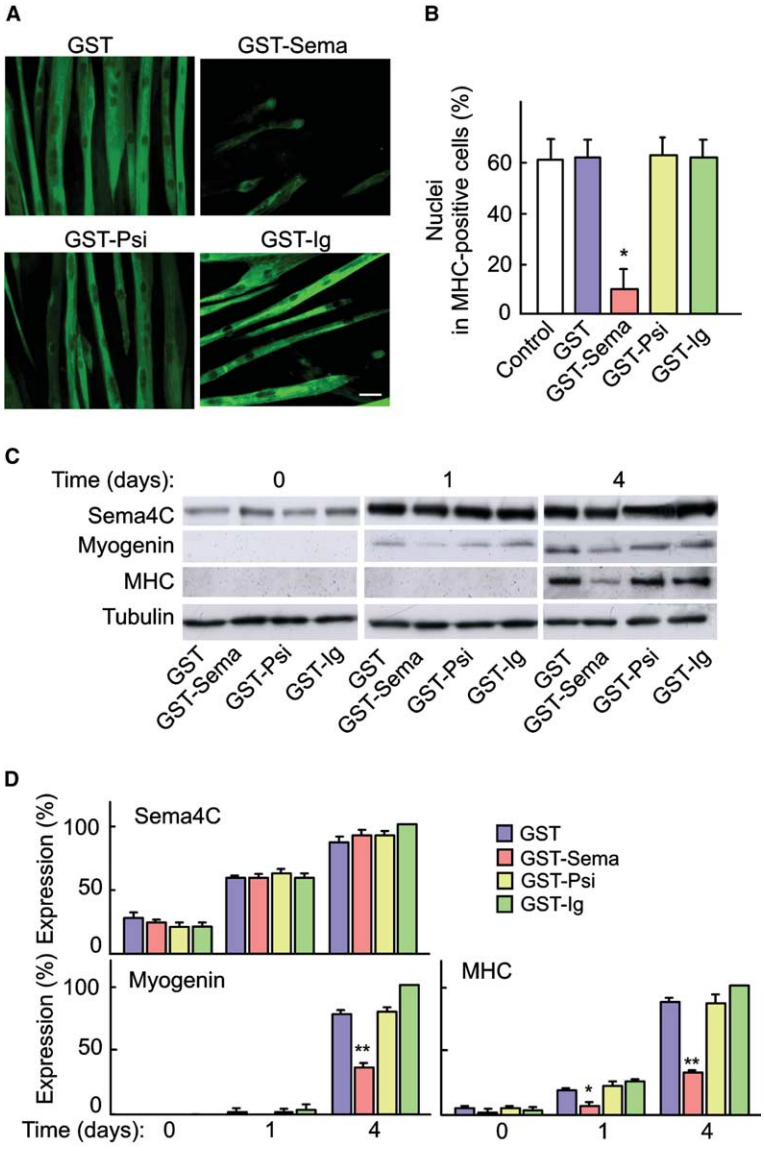

Fig. 3. Inhibition of myoblast differentiation by a fusion protein containing the extracellular Sema domain of Sema4C. (A) C2C12 cells were cultured in the absence (control) or presence of GST, GST-Sema, GST-Psi, or GST-Ig (2.5 μg/ml) for 5 days after the induction of differentiation. The cells were then immunostained with antibodies to MHC. Bar, 25 μm. (B) Nuclei were visualized with DAPI. The percentage of nuclei in MHC-positive cells in each of 10 randomly chosen fields (magnification, 200×) was determined at this time to quantify myotube formation. Data are means ± S.E. from a representative experiment; $^{*}P < 0.001$ versus control. (C) Cell lysates were subjected to immunoblot analysis at the indicated times after induction of differentiation. (D) The expression of each protein was quantified by scanning blots similar to those shown in (C) was normalized by the expression level of tubulin, and was expressed as a percentage of the maximum normalized value. Data are means ± S.E. from three independent experiments; $^{*}P < 0.05$, $^{**}P < 0.01$ versus GST.

protein within the cell might be important for the induction of myoblast differentiation. We exposed C2C12 myoblasts to a synthetic peptide (TAT-W) containing the COOH-terminal 13 amino acids of Sema4C linked to a modified TAT sequence of 11 amino acids that mediates the transport of heterologous peptides into mammalian cells [13]. The addition of this peptide to DM inhibited the formation of myotubes (Fig. 4A and B) but it had no marked effect on the expression of Sema4C or myogenin (Fig. 4C). A control peptide (TAT-M) in which the PDZ domain-binding motif was mutated (ASA) had no effect on the differentiation of C2C12 cells (Fig. 4). These results thus implicated the PDZ domain-binding motif of Sema4C in myogenic differentiation.

3.5. Up-regulation of Sema4C during injury-induced muscle regeneration in vivo

To determine whether Sema4C participates in skeletal muscle differentiation in vivo, we examined its expression in a mouse model of muscle regeneration [14,16]. Muscle damage was induced by intramuscular injection of cardiotoxin, and injury-induced muscle regeneration was monitored morphologically. As shown in Fig. 5A, regeneration was proceeding normally. As described previously [16], MyoD and myogenin were undetectable in uninjured muscle but were detected 3 days after injury, gradually returning to basal levels thereafter (Fig. 5B). Although Sema4C was expressed at a low level before injury, its abundance was also markedly increased 3 days

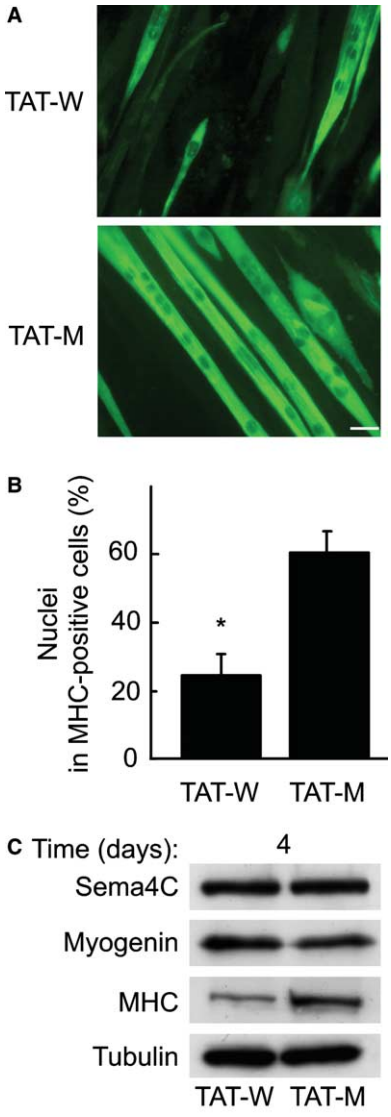

Fig. 4. Inhibition of myoblast differentiation by a synthetic peptide containing the COOH-terminal region of Sema4C. (A) C2C12 cells were cultured in the presence of a synthetic peptide containing wild-type (TAT-W) or mutated (TAT-M) versions of the COOH-terminal sequence of Sema4C (10 μM) for 24 h before and for 4 days after the induction of differentiation by culture in DM. The cells were then immunostained with antibodies to MHC. Bar, 25 μm. (B) Nuclei were visualized with DAPI. The percentage of nuclei in MHC-positive cells in each of 10 randomly chosen fields (magnification, 200×) was determined to quantify myotube formation. Data are means ± S.E. from a representative experiment; $^{*}P < 0.001$ versus the corresponding value for cells treated with TAT-M. (C) Cell lysates prepared 4 days after the induction of differentiation were subjected to immunoblot analysis.

after injury, returning to basal levels by 9 days. These results thus suggest that Sema4C contributes to terminal myogenic differentiation in vivo.

4. Discussion

The transmembrane semaphorin Sema4C is expressed in a wide variety of tissues in a developmentally regulated manner [8]. However, the functions of this protein have remained unknown. We have now shown that both the early stage of differentiation of C2C12 mouse skeletal myoblasts into myotubes and injury-induced muscle regeneration in vivo are accompanied by up-regulation of Sema4C. The differentiation-associated increase in the expression of Sema4C in C2C12 cells precedes that of the myogenic transcription factor myogenin. The inhibitory effects both of Sema4C depletion by RNAi and of a GST fusion protein containing the extracellular Sema domain of Sema4C revealed that Sema4C signaling contributes to the induction of myogenin expression and myotube formation in C2C12 cells. Sema4C thus plays an essential role in the early steps of myogenic differentiation of myoblasts into myotubes. The regeneration of skeletal muscle after injury occurs by a process that is highly similar to embryonic muscle differentiation [17]. The up-regulation of Sema4C as well as MyoD and myogenin observed during injury-induced muscle regeneration suggests that induction of myogenin expression and myotube formation by Sema4C occurs not only in cultured cells but also in vivo.

Members of two protein families, plexins and neuropilins, function as receptors to mediate the actions of semaphorins in axon guidance [9,10]. Plexins alone are able to bind membrane-bound semaphorins, whereas plexin–neuropilin complexes act as high-affinity receptors for secreted semaphorins. The signaling pathway downstream of plexins is largely unknown, although small GTPases and tyrosine phosphorylation have been implicated in growth cone collapse [10]. Although the receptor responsible for mediating the action of Sema4C in terminal myogenic differentiation remains to be identified, our results clearly demonstrated that the intercellular interaction of myoblasts mediated by Sema4C through the Sema domain plays a key role in terminal myogenic differentiation by up-regulating myogenin.

Transmembrane semaphorins are thought to mediate bidirectional signaling [18]. Our results now indicate that outside-in signaling by Sema4C through its intracellular COOH-terminus is also important for terminal myogenic differentiation. This signaling through the COOH-terminus of Sema4C does not affect the induction of myogenin, however, whereas signaling by the extracellular Sema domain through its receptor controls the expression of myogenin. Interaction of Sema4C with the PDZ domains of PSD-95 family proteins is thought to occur in the brain [19]. Association of Sema4C with the PDZ domain-containing proteins SEMCAP1 (GIPC) and SEMCAP2 has also been suggested [20]. The putative PDZ domain-containing protein that interacts with the COOH-terminus of Sema4C as well as the downstream signaling proteins in myoblasts await identification.

A GST fusion protein containing the Sema domain of Sema4C inhibited both the formation of myotubes and the up-regulation of myogenin, probably by competing with Sema4C for its receptor. Disulfide bonds in the extracellular region of semaphorins are thought to be essential for the structure and biological activity of these proteins [21–23]. The GST-Sema fusion protein used in the present study was expressed in *E. coli* and therefore might not be expected to contain the normal pattern of disulfide bonds. Although it was not able to reproduce the activity of Sema4C in C2C12 cells, likely because of an improper conformation, GST-Sema was able to inhibit the interaction between Sema4C and its receptor at relatively high

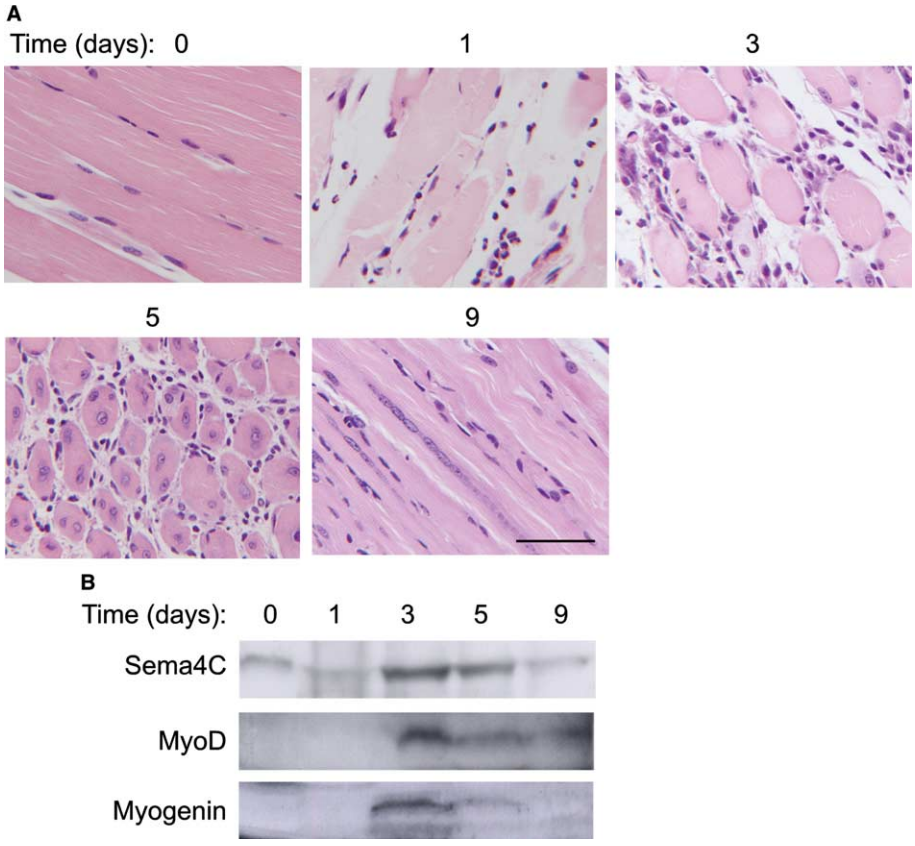

Fig. 5. Expression of Sema4C during regeneration of damaged mouse skeletal muscle. (A) Muscle injury and regeneration were induced by intramuscular injection of cardiotoxin into the hind limb, and sections of muscle isolated before or 1, 3, 5, or 9 days after injury were stained with hematoxylin–eosin. Bar, 50 μm. (B) Homogenates of muscles isolated at the indicated times after injury were subjected to immunoblot analysis.

concentrations. Indeed, whereas the concentration of GST-Sema used in our experiments was ~30 nM, the affinity of the transmembrane semaphorin Sema4D for its plexin receptor was previously found to be 0.9 nM [24].

Cell adhesion molecules including cadherins [5,25–31] as well as immunoglobulin superfamily members such as CDO and BOC [32–34] play important roles in skeletal muscle myogenesis. The effects of Sema4C in myoblasts revealed in the present study are similar to those of cadherins. Cadherin-mediated adhesion promotes the formation of adherens junctions, the recruitment of β-catenin to sites of cell–cell contact, and the induction of myogenin expression and myotube formation. Both cadherins [5] and Sema4C increase myogenin expression independently of MyoD, whereas CDO does not affect myogenin expression [32]. Both Sema4C and β-catenin also contain a PDZ domain-binding motif in their COOH-terminal regions. It will thus be of interest to examine the localization of Sema4C in myoblasts in relation to that of adherens junctions. The available antibodies to Sema4C are not suitable for immunostaining, however. Recently, the axon guidance molecule netrin and its receptor, neogenin, were shown to stimulate myotube formation [34]. Cadherins and immunoglobulin superfamily members including CDO, BOC, and neogenin together with its ligand netrin have been proposed to form a large complex at the cell surface [34,35]. It is possible that Sema4C and its associated proteins are also components of this complex.

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